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14. ABSTRACT

This project is designed to test the hypothesis that chronic exposure to depleted uranium (DU) impairs neuronal processes underlying cognitive function via alterations induced at hippocampal glutamatergic synapses. Evidence has been uncovered that suggests a direct effect of uranium to diminish stimulated hippocampal glutamate release, which may account for the reported decrease in neuronal excitability (3). However, other findings indicate that long-term DU exposure may also inhibit glial glutamate and GABA uptake and up-regulate NMDA receptors, possibly predisposing the individual to excitotoxicity and neurodegenerative disease. The establishment of the DU chronic exposure protocol as a shrapnel wound model based on blood and brain levels of the metal and altered rates of growth is also notable. These measures provide benchmark values for future studies and for correlation of results from this project to those obtained in other laboratories. Given the similarity of the effects of uranium on transmitter release to those of other multivalent metals (e.g., methylmercury, lead) and the fact that exposure in military scenarios is continuing, it is clear that additional studies are warranted on uranium's actions, particularly those related to developmental neurotoxicity.

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depleted uranium, glutamate release, military disease, NMDA receptor, hippocampus, tissue slice perfusion

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INTRODUCTION

The chemical properties of depleted uranium (DU) render the metal well suited for military purposes. The U. S. Army utilizes DU for tank armor and for kinetic energy penetrators in munitions, and has deployed such weapons in the 1st Gulf War, in Kosovo, and in Iraq. Use of the metal in future military arenas is a virtual certainty, but knowledge of its neurotoxicity is lacking. Gulf War veterans who retained fragments of DU shrapnel over several years have exhibited lowered performance on neurocognitive tests (1). Research studies in chronically exposed rats have shown that kidney and tibia had the highest concentrations of the metal, but that significant levels accumulated in other tissues such as the brain, suggesting the potential for CNS consequences of exposure to DU via this route (2). Moreover, other studies in chronically exposed animals indicated alterations in hippocampal synaptic transmission, suggesting DU-induced decreases in neuronal excitability (3). More extensive knowledge is needed therefore of the nature of DU-induced neurotoxicity, particularly with respect to developing improved therapeutic approaches for treatment of exposed military personnel. *This research proposal therefore tested the overall hypothesis that chronic exposure to DU impairs neuronal processes underlying cognitive function via alterations induced at hippocampal glutamatergic synapses that directly modulate Ca^{+2}-mediated cellular processes.* Glutamatergic function has been assessed in rats exposed chronically via intramuscular implants of varying amounts of DU pellets in order to identify the bases for the impaired cognition and diminished neuronal excitability. Components of depolarization-evoked glutamate release have been measured in the presence of acute *in vitro* or after extended *in vivo* exposure to the metal (Technical Objective 2). Determination of the actions of uranium on glutamatergic NMDA receptors has been performed via approaches employing chronic *in vivo* exposures (Technical Objective 3). Other studies determined the concentrations of DU produced in blood and brain tissue as a result of exposure (Technical Objective 1). These results will be of critical importance to U. S. armed forces in defining risk and establishing treatment modalities for DU exposures sustained in recent conflicts and in future battlefield situations.

BODY

The following sections provide a comprehensive and complete record of the research accomplishments of the project relative to the Statement of Work and Technical Objectives. Presentation of the results is organized according to the outline of the investigation described in these latter documents.

Blood and Brain DU Concentrations

The initial studies in this line of investigation were performed to characterize the pellet implant exposure protocol to be utilized in subsequent work by determining blood and brain tissue concentrations. Rats were exposed to 0 (controls), 300, or 600 mg DU by implantation of 30-mg (2 mm × 1 mm diameter cylinders) pellets in the gastrocnemius muscles of their hindlimbs. Tantalum pellets of the same size were used in control animals and to balance the total metal mass implanted across groups with an inert metal. Male Sprague-Dawley rats, 70-100 days of age, were anesthetized, and each foot was wrapped in sterile gauze and the leg surrounded by a sterile drape. A 2.0 cm incision was made in the leg until the gastrocnemius muscle was visualized. Pellet implantation began by inserting the tip of an 18-gauge needle into the incision.

A 16-gauge Rosenthal needle was then positioned 2 mm down into the muscle and the pellet inserted via the needle plunger. The remaining 9 pellets were inserted in the same manner approximately 1.0 mm apart. The incision was closed and a triple antibiotic ointment was applied to the wound. Ampicillin (100,000 IU/kg) was given intramuscularly, and the rat placed under heat until awakening. Animals were anesthetized again after exposure periods of 1, 3, 6, or 12 months (N = 6/group/time period), and 1 ml of blood collected by cardiac puncture and placed into heparinized tubes. The rats were then sacrificed and hippocampus was removed, weighed, and stored at -20ºC. Prior to implantation pellets were chemically cleaned by brief soaking in 50% nitric acid, rinsing with distilled water, and drying in a stream of argon. Pellets were stored under argon until use, and briefly immersed in isopropyl alcohol just prior to placement. Analyses were performed by Elemental Analysis Inc. (Lexington, KY) with a VG PlasmaQuad 3 Quadrapole ICP-MS.

Blood uranium concentrations in chronically exposed animals increased monotonically as a function of length of exposure ($F = 26.87$, $p < 0.001$), and did so in an exposure level-dependent fashion ($F = 110.27$, $p < 0.001$; Figure 1). A significant interaction ($F = 10.57$, $p < 0.001$) indicated that the increases in the metal over time varied across groups. DU group means were significantly higher than tantalum control values at each duration interval at $p < 0.001$, while the high dose group means were higher than the low dose values as shown in the Figure. Values in the high DU group reached ~3.0 ng/ml after 12 months' exposure.

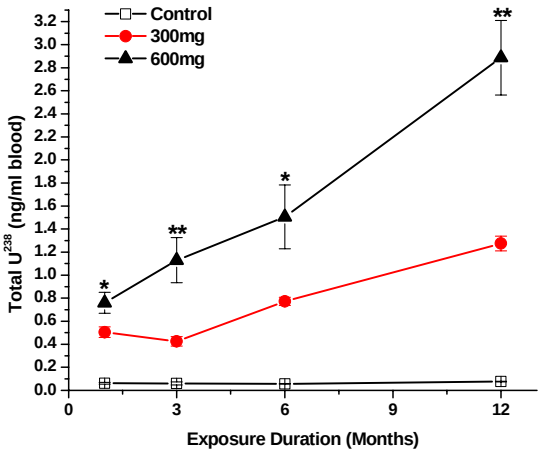

Figure 1. Concentrations of uranium in rat whole blood as a function of amount and duration of exposure to implanted DU pellets as determined by ICP-MS. Values are expressed as mean ± SEM with N = 6 for each exposure group and duration. *** p < 0.001; ** p < 0.01; * p < 0.05 compared to 300 mg values at the same exposure duration.

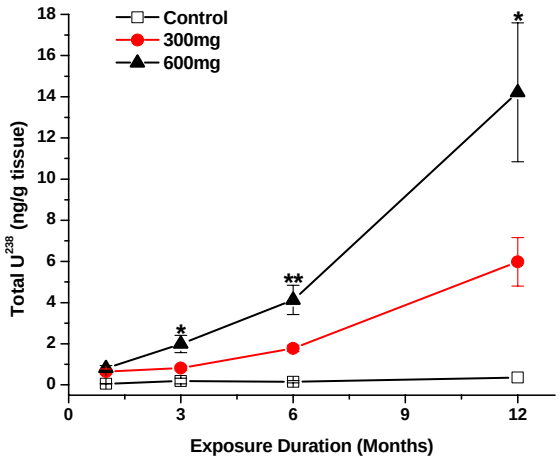

Figure 2. Concentrations of uranium in rat hippocampus as a function of amount and duration of exposure to implanted DU pellets as determined by ICP-MS. Values are expressed as mean ± SEM with N = 5-6 for each exposed group and duration. *** p < 0.001; ** p < 0.01; * p < 0.05 compared to 300 mg values.

Analogous values for DU concentrations in hippocampal tissue are shown in Figure 2 as a function of exposure level and duration. Similar to the blood levels, uranium concentrations in chronically exposed animals increased monotonically up to 12 months duration ($F = 8.18$, $p < 0.001$) in an exposure level-dependent fashion ($F = 12.17$, $p < 0.001$). A significant interaction ($F = 3.53$, $p < 0.01$) indicated that the increases in the metal over time varied across groups. DU group means were significantly higher than tantalum control values at each duration interval at p

values < 0.05 or lower, while the high dose group means were higher than the low dose values as shown in the Figure. Values in the high dose group climbed to ~14 ng/g tissue after 12 months exposure. It is important to note the consistently low DU concentrations measured in control blood and tissue – these values are indicative of the cleanliness of the sample collection and analytical procedures. Contamination of control samples has been readily observable in some other published works employing chronic DU exposure (e.g., 4-5).

In addition, body weights of all male littermates were recorded at 4-week intervals during exposure, and several criteria were adapted to guide the statistical analyses and insure the validity and integrity of these findings. Since the capacity for somatic growth varies as a function of age and since age at the time of DU pellet implantation was not uniform across groups, a range for pre-implantation body weights was identified to insure similar potential for growth. Thus, only animals weighing at least 250 g at the time of implantation surgery but not more than 400 g were included in the analyses. This criterion resulted in the inclusion of 15-19 growth records for each exposure group. A linear mixed model was chosen for the analysis to permit data to exhibit correlated and non-constant variability, and so that growth records utilizing unequal intervals or having missing values could be included in the analyses (6).

Somatic growth for each group as a function of exposure duration is summarized in Figure 3. The test of fixed effects within the linear mixed model uncovered a significant effect of exposure ($p = 0.003$), manifested as a diminished rate of growth in the both DU groups. Compared to the control tantalum pellet group, the 300 mg ($p = 0.001$) and 600 mg ($p = 0.025$) DU groups both exhibited diminished rates of growth. The impaired rate of growth observed in this work is remarkably similar to that reported by Pellmar *et al.* (7) who used a similar exposure regimen.

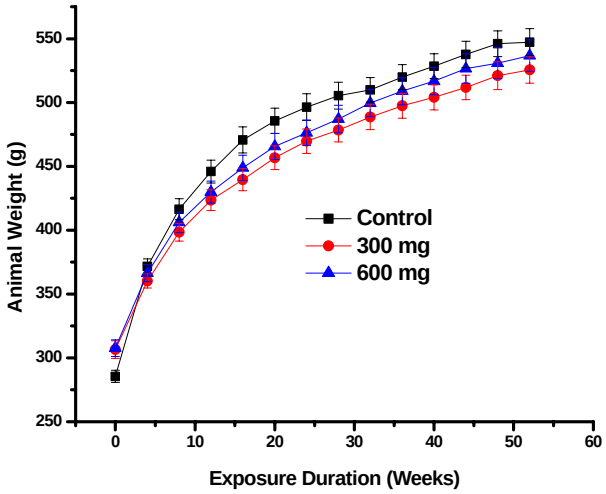

Figure 3. Increases in whole rat body weight as a function of amount and duration of exposure to implanted DU pellets. Control and low dose (300 mg) DU groups received tantalum pellet implants so that all animals received the same total mass of metal. Data were evaluated using a linear mixed model comparing growth rate across exposure groups using pre-implantation body weight as a starting point. Values are expressed as mean ± SEM with N = 15-19 for each exposed group and duration. An overall significant decrease in body weight was uncovered in both DU groups.

Acute DU Exposure and Glutamate/GABA Release

The acute exposure studies were designed to identify the direct effects of uranium on the exocytotic process, and utilized a superfusion procedure that permits measurement of endogenous glutamate and GABA release from hippocampal synaptosomes. A range of uranium concentrations was applied to the synaptosomes via the superfusing buffer to permit estimation of the inhibitory/stimulatory potency on the transmitter release component under study.

Hippocampi from 60-70 day old male Sprague Dawley rats were collected and homogenized in a 10 mM HEPES-0.32M sucrose buffer (pH 7.4). The homogenate was centrifuged for 2 min at 3000×g_{max}, and the supernatant transferred to a clean tube, and centrifuged again for 12 min at 14,600×g_{max}, resulting in isolation of a synaptosomal pellet. The pellet was resuspended in HEPES-sucrose buffer and synaptosomes diluted to 1.0 mg protein/ml with an isotonic HEPES buffer (containing in mM: NaCl 132, KCl 1, $MgCl_2$ 1, $CaCl_2$ 0.1, glucose 10, HEPES 10, and 0.1% bovine serum albumin (BSA); bubbled in 99.9% O_2, pH 7.40) and incubated for 30 min at 37^{o}C. Carbonate and phosphate salts were not used in the buffer because of the ready formation of uranyl precipitates at this pH.

The synaptosomes were centrifuged and resuspended to 4.0 mg/ml in the above HEPES-buffer (without BSA, and containing a glutamate reuptake blocker - 0.5 mM DL-threo-β-hydroxyaspartic acid). Exposure solutions also contained uranium oxynitrate at concentrations ranging from $10^{-3.5}$ to 10^{-8} M. 800 mg of synaptosomal protein were added to superfusion chambers, and flow initiated with the same HEPES buffer at 0.6 ml/min. Flow was maintained for 30 min and then baseline samples were collected at 2 min intervals. During perfusate collection superfusion was switched for 2 min to the same HEPES buffer containing 31 mM KCl (Na^+ reduced to maintain isotonicity) and then returned to the normal buffer to re-establish the baseline. After superfusion was complete, samples were prepared for HPLC analysis by derivatization using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. The derivatives were analyzed using binary gradient liquid chromatography with fluorescence detection (excitation - 250 nm and emission - 395 nm).

The presence of uranium oxynitrate in the superfusion buffers diminished depolarization-evoked synaptosomal glutamate release across the range of metal concentrations tested. Figure 4 shows the time course of the effects of 10 µM uranium on stimulated glutamate release compared to

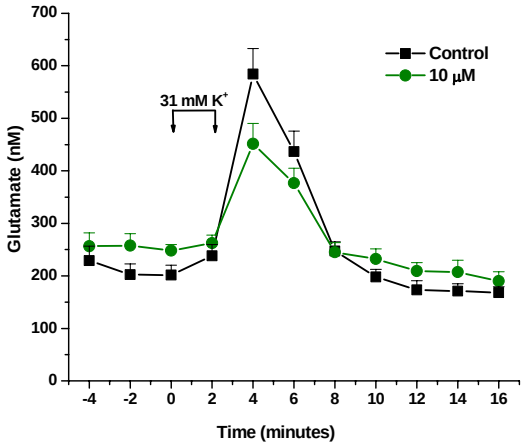

Figure 4. Time course of glutamate concentration in response to superfusion with 31 mM K^+ across hippocampal synaptosomes in standard 10 mM HEPES-sucrose buffer (pH 7.4) or in buffer containing 10 µM uranium. The stimulation-evoked increase in endogenous glutamate was diminished by the presence of uranium. Values are expressed as mean ± SEM of independent determinations – N = 10 for control conditions, and 4-5 for each uranium concentration) – conducted in triplicate.

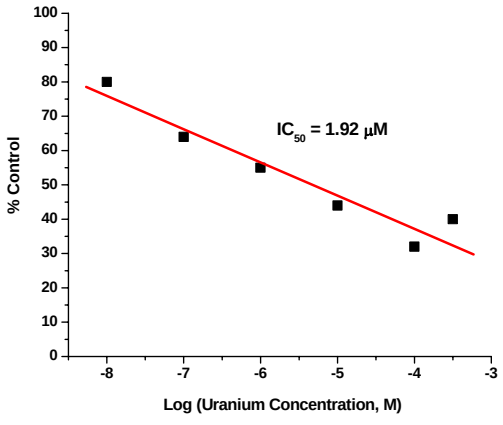

Figure 5. Percentage inhibition of stimulated glutamate release from hippocampal synaptosomes as a function of the concentration of uranium in the superfusing solution. Maximal inhibition is limited by the solubility of uranium in aqueous media at pH 7.4. The data were fitted by linear regression of the types of response inhibition shown in Figure 4 using Simpson's Rule to compute areas under the response curve.

that from a non-exposed (i.e., control) preparation. The high K^+ stimulus elicited a maximal response that represented an approximate 3-fold increase in non-exposed synaptosomes, while smaller increases were seen in the presence of superfused uranium. The areas under the stimulation response curves were computed using Simpson's rule (8) and compared across uranium concentrations to the control area, resulting in a percent inhibition of the response for each level of uranium. These transformations result in the inhibition regression line for stimulated glutamate release shown in Figure 5.

An analogous effect on the time course of K^+-stimulated hippocampal GABA release is shown in Figure 6. Again, the maximal response is an approximate 3-fold increase in release with smaller responses observed in the presence of concentrations of uranium. An inhibition regression line for the effect of uranium on stimulated GABA release is shown in Figure 7. The effect of acute exposure to the metal is strikingly less potent with respect to GABA release (IC_{50} = 2.59 mM) than it is for glutamate release (IC_{50} = 1.92 µM).

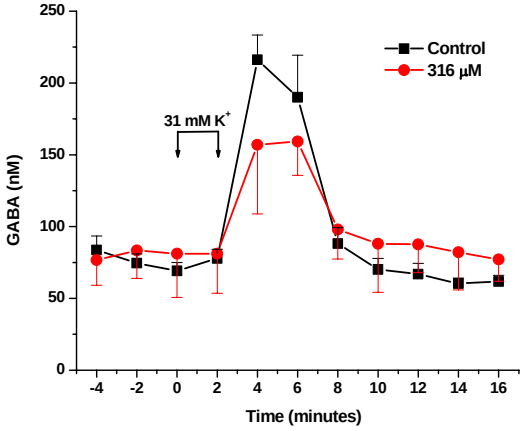

Figure 6. Time course of GABA concentration in response to superfusion with 31 mM K^+ across hippocampal synaptosomes in standard 10 mM HEPES-sucrose buffer (pH 7.4) or in buffer containing 316 µM uranium. The stimulation-evoked increase in endogenous GABA was diminished by the presence of uranium. Values are expressed as mean ± SEM of independent determinations – N = 10 for control conditions, and 4-5 for each uranium concentration – conducted in triplicate.

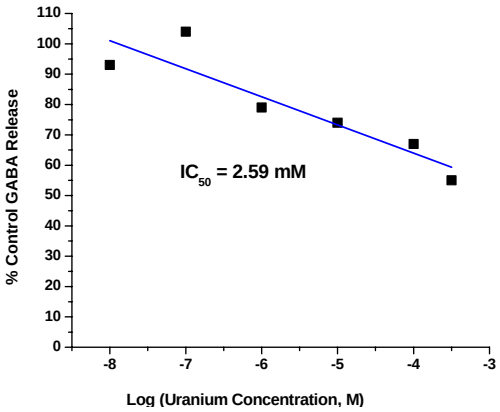

Figure 7. Percentage inhibition of stimulated GABA release from hippocampal synaptosomes as a function of the concentration of uranium in the superfusing solution. GABA release is much less sensitive to acute uranium exposure than that of glutamate. Maximal inhibition is limited by the solubility of uranium in aqueous media at pH 7.4. The data were fitted by linear regression of the types of response inhibition shown in Figure 6.

The basis for the differential potency of uranium on glutamate and GABA release is not known. GABA peaks are smaller, occur later in the chromatogram, and are somewhat susceptible to co-elution with other unknown compounds. But no chromatographic factors have been found to contribute to this differential sensitivity. It is possible that uranium binds glutamate (9) – but not GABA – in a soluble complex, but if this occurs it does not diminish the derivatization efficiency for glutamate. Each set of samples from an experimental run included triplicate sets of standards derivatized in the presence of the concentrations of uranium used in that assay. No uranium effect on derivatization could be discerned, and thus such a mechanism cannot account for the observed differential potency. Alternatively, it is plausible that uranium could complex glutamate in such a fashion as not to affect derivatization but that might more potently bind to synaptic sites related to transmitter release, e.g., to block voltage-sensitive Ca^{+2} channels. Elucidation of the mechanism(s) involved will require further investigation.

The uranium IC_{50} for glutamate release of 1.92 µM based on nominal metal concentrations is remarkably similar to the inhibitory potencies of a number of other multivalent metal ions (10). This suggests by inference that the action of uranium is exerted at membrane voltage-sensitive Ca^{+2} channels to interfere with Ca^{+2} influx and diminish exocytosis. Moreover, some of these multivalent metals (e.g., lead) are well known to possess developmental neurotoxicant properties.

The acute *in vitro* exposure studies also have assessed the Ca^{+2}-mimetic properties of uranium by measuring synaptosomal depolarization-evoked transmitter release in the absence of Ca^{+2} (replaced by Mg^{+2} to maintain perfusion medium isotonicity) and in the presence of a Ca^{+2} channel antagonist (methoxyverapamil) in the superfusion buffer. Preliminary studies showed that this component of glutamate release constituted only ~25-30% of the total release observed under the conditions described above. If uranium at least partially supports exocytosis – like Pb^{+2} – then this release component should be greater than that found in non-exposed preparations.

Figure 8 displays the time course of K^+-stimulated glutamate release in the absence of Ca^{+2} in control hippocampal synaptosomes, reflecting an approximate 50% increase over baseline values. This compares with a three-fold increase observed in the presence of Ca^{+2} in the superfusion medium as shown in Figure 4 above. However, the data shown in Figure 9 indicate that there is no observable change in the magnitude of this release across a range of nominal superfused uranium concentrations. Similarly, Figure 10 displays the time course of K^+-stimulated GABA release in the absence of Ca^{+2} in non-exposed hippocampal synaptosomes, reflecting a response approximately 60% of that achieved in the presence of Ca^{+2} as shown in Figure 6 above. Again, the data shown in Figure 11 indicate that there is no observable change in the magnitude of this release across a range of nominal superfused uranium concentrations.

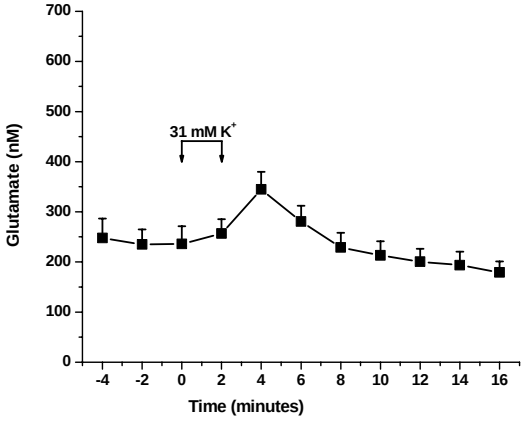

Figure 8. Time course of glutamate concentration in response to superfusion with 31 mM K+ across hippocampal synaptosomes in calcium-free 10 mM HEPES-sucrose buffer (pH 7.4) containing a calcium channel antagonist. Stimulation evoked a peak increase in endogenous glutamate of 44% over baseline concentrations. Values are expressed as mean ± SEM of independent determinations - N = 8 for control conditions and 3-4 for each uranium concentration - conducted in triplicate.

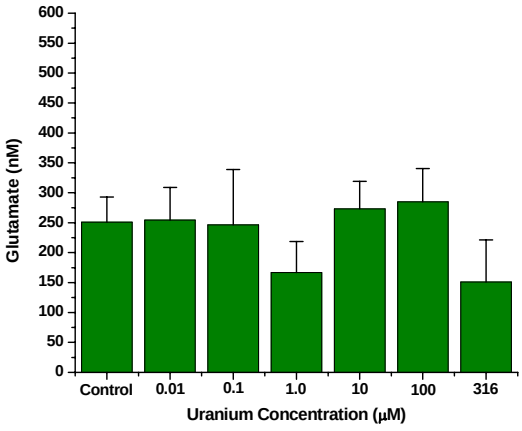

Figure 9. Magnitude of the stimulated calcium-free glutamate responses to 31 mM K+ as in Figure 8 across a range of perfusate uranium concentrations. There were no significant differences in calcium-independent glutamate release from control values at any exposure level. Means are expressed + SEM of independent determinations - N = 6 for control conditions and 3 for each uranium concentration - conducted in triplicate.

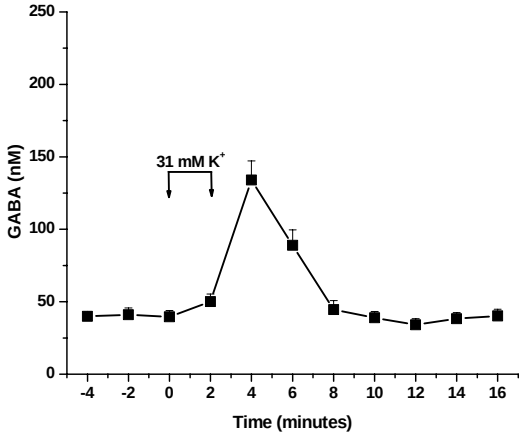

Figure 10. Time course of GABA concentration in response to superfusion with 31 mM K+ across hippocampal synaptosomes in calcium-free 10 mM HEPES-sucrose buffer (pH 7.4) containing a calcium channel antagonist. Stimulation evoked a peak 3.3-fold increase in endogenous GABA over baseline concentrations. Values are expressed as mean + SEM of independent determinations - N = 8 for control conditions and 3-4 for each uranium concentration - conducted in triplicate.

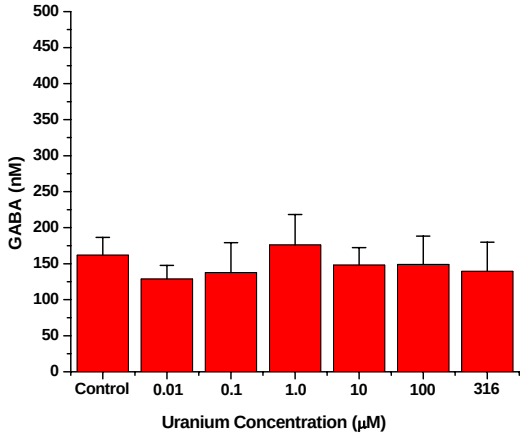

Figure 11. Magnitude of the stimulated calcium-free GABA responses to 31 mM K+ as in Figure 10 across a range of perfusate uranium concentrations. There were no significant differences in calcium-independent GABA release from control values at any exposure level. Means are expressed ± SEM of independent determinations - N = 8 for control conditions and 3-4 for each uranium concentration - conducted in triplicate.

In summary, depolarization of superfused hippocampal synaptosomes with high K^+ in Ca^{+2}-free buffer evoked modest increases in glutamate and GABA release, but acute uranium exposure *in vitro* across a range of concentrations did not significantly or systematically alter these response magnitudes. This suggests that UO_2^{+2} does not possess Ca^{+2}-mimetic properties, but it could also be explained if the intrasynaptosomal UO_2^{+2} concentrations did not achieve sufficient levels during the acute exposure to manifest such an effect. The uranium species involved in the effect on glutamate exocytosis is not known. Uranyl ion (UO_2^{+2}) – the most common form produced in the body from all forms of the metal – is converted to diuranate ion ($U_2O_7^{-2}$) under alkaline conditions (11), so this or another uranium complex (e.g., UO_2OH^+, 12) may be involved.

Chronic DU Exposure and Glutamate/GABA Release

The studies utilizing a chronic exposure regimen were conducted to identify the effects of the metal with the assumption that the results would represent a combination of the direct actions of DU along with associated and independent compensatory and long-term effects. The results from the acute exposure experiments would be incorporated into interpretation of the chronic exposure observations to enhance identification of the synaptic processes involved. Rats were exposed to 0, 300, or 600 mg DU by implantation of 30-mg pellets in their hindlimbs as described above, and exposure maintained for 15-17 months. At the conclusion of this period animals were sacrificed and brains removed. Transverse dorsal hippocampal slices were cut 400 µm thick in a vibratome and collected in oxygenated HEPES-sucrose buffer (in mM: NaCl 132, KCl 1, $CaCl_2$ 2.6, HEPES 10, sucrose 10, pH 7.6) at 4° C. Slices were then incubated in the same buffer for 1-2 hr at 33°C. Three slices (~175-200 µg total protein) were gently placed on filters in each chamber of a superfusion apparatus. Superfusion was initiated with the same HEPES buffer at 0.6 ml/min. Flow was maintained for 40 min and then baseline samples were collected at 2 min intervals. During perfusate collection, the solution was switched for 2 min to 50 mM KCl buffer as described above (K^+ replacing Na^+ to maintain isotonicity) containing a

glutamate reuptake blocker - 0.5 mM DL-threo-hydroxyaspartic acid - and then returned to the starting buffer to re-establish the baseline. To assess Ca^{+2}-independent release Ca^{+2} was replaced in the buffer by Mg^{+2} (final concentration 2.3 mM) and a Ca^{+2} channel antagonist (300 µM methoxyverapamil) added. After superfusion was complete, samples were prepared for HPLC analysis by derivatization and quantified as described earlier for synaptosomal fractions. At the conclusion of the experiment slices were retrieved from the chamber filters and sonicated for determination of total protein using the bicinchoninic acid method (13). The protein values were then used to normalize glutamate/GABA concentrations across chambers.

Figure 12 displays the time course of the response of hippocampal glutamate release to high K^+ in the presence of Ca^{+2} in the superfusion medium. In contrast to the effects of acute uranium exposure, chronic DU produced an **increase** in the response of glutamate release up to 8 minutes after initiation of depolarization. The effect was most apparent at the 600 mg DU dose, but also could be seen in the 300 mg group near the beginning of high K^+ perfusion. Then Ca^{+2} was replaced by Mg^{+2} in the superfusion medium and a voltage-sensitive Ca^{+2} channel antagonist added to substantially eliminate Ca^{+2} influx in order to again explore the possible presence of DU's Ca^{+2}-mimetic properties. As shown in Figure 13 the time course of the response under Ca^{+2}-free perfusion conditions exhibited a smaller but nonetheless statistically significant enhanced glutamate response to high K^+ at the high DU exposure level.

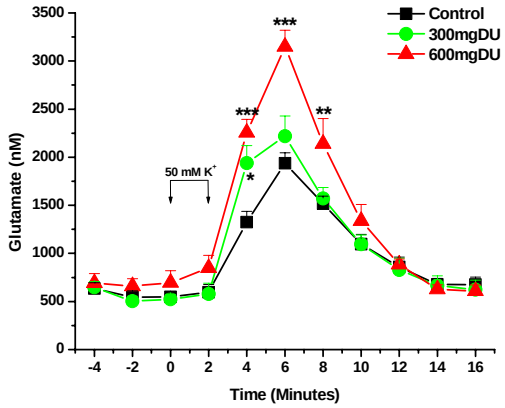

Figure 12. Time course of glutamate concentration in response to superfusion with 50 mM K^+ across transverse hippocampal slices in standard 10 mM HEPES-sucrose buffer (pH 7.4). Values are expressed as mean ± SEM based on 8 animals/group with sample determinations conducted in triplicate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ relative to the glutamate concentration in control animals at the same time point.

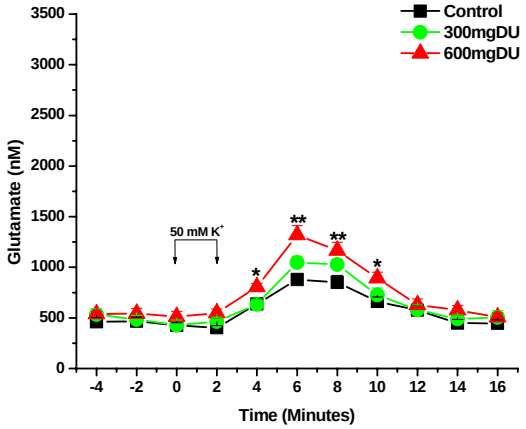

Figure 13. Time course of glutamate concentration in response to superfusion with 50 mM K^+ across transverse hippocampal slices in standard 10 mM HEPES-sucrose buffer (pH 7.4) in the absence of Ca^{+2}. Values are expressed as mean ± SEM based on 6-8 animals/group with sample determinations conducted in triplicate. *$p < 0.05$; **$p < 0.01$ relative to the glutamate concentration in control animals at the same time point.

In contrast to the relative absence of an effect of acute uranium exposure, chronic DU resulted in an increase in the response of hippocampal GABA release to depolarization in the presence of Ca^{+2} as shown in Figure 14. The time course of the enhanced response was proportionally similar to that of glutamate (see Figure 12), but was observable only in the 600 mg DU group. When Ca^{+2}-free medium was superfused as above, high K^+ evoked statistically significant changes in GABA concentration in the high dose group that were very similar to those exhibited by glutamate as displayed in Figure 15.

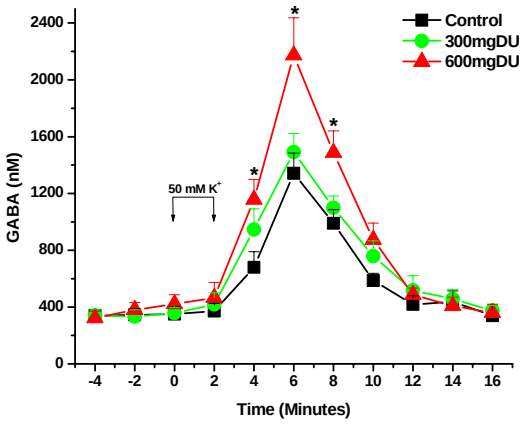

Figure 14. Time course of GABA concentration in response to superfusion with 50 mM K^+ across transverse hippocampal slices in standard 10 mM HEPES-sucrose buffer (pH 7.4). Values are expressed as mean ± SEM based on 8 animals/group with sample determinations conducted in triplicate. *$p < 0.05$ relative to the GABA concentration in control animals at the same time point.

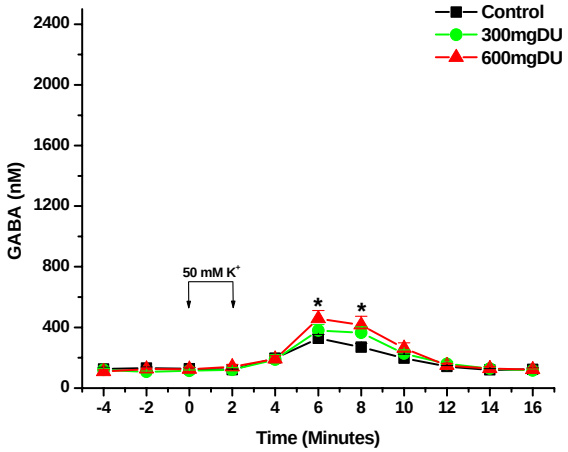

Figure 15. Time course of GABA concentration in response to superfusion with 50 mM K^+ across transverse hippocampal slices in standard 10 mM HEPES-sucrose buffer (pH 7.4) in the absence of Ca^{+2}. Values are expressed as mean ± SEM based on 6-8 animals/group with sample determinations conducted in triplicate. *$p < 0.05$ relative to the GABA concentration in control animals at the same time point.

The increase in stimulated transmitter release found after chronic DU exposure is in contrast to the effects of acute exposure *in vitro*, where divalent uranyl ion was found to diminish the magnitude of depolarization-evoked glutamate release, and to reports of decreased hippocampal neuronal excitability (2). There is no evidence suggesting the presence of substantial calcium-mimetic properties for uranium. Such an effect was not observed after acute exposure *in vitro* (Figures 9 and 11), and though stimulated release was increased in the absence of Ca^{+2} (Figures 13 and 15), the magnitude of the increase was much less than that seen in the presence of Ca^{+2} (Figures 12 and 14), the opposite relationship to be expected if significant Ca^{+2}-mimetic properties were present (see e.g., 14). Alternatively, the enhancement in stimulated transmitter release may be due to uptake of the metal into astrocytes, disruption of mitochondrial function, and consequent inhibition of glutamate/GABA uptake, similar to actions reported for methylmercury (e.g., 15-16). **Therefore, throughout the rest of this report the term 'release' will be applied to measured slice superfusate neurotransmitter concentrations, though the term may not precisely represent the amount of stimulated exocytosis because of uptake inhibition.** Further studies are necessary to test this hypothesis.

Chronic DU Exposure and NMDA Receptors

This study was conducted to assess NMDA receptor function after extended exposure to DU. ^{3}H-MK-801 binding is established as a measure of accessibility or activation of the NMDA receptor-regulated ion channel. Rats were exposed to 0, 300, or 600 mg DU by implantation of 30-mg pellets in their hindlimbs as described above, and exposure maintained for 15-17 months. A crude synaptic membrane fraction was prepared by homogenization of dissected hippocampal tissue in 40 vol of 50 mM Tris acetate (pH 7.4) followed by centrifugation at 50,000*g* for 20 min. The resulting pellet was resuspended in the same volume of buffer and the centrifugation-resuspension cycle repeated two more times. The final resuspension was in 8 vol of 0.32 M sucrose with the preparation then frozen at –80^o C until use. Protein concentration was

determined by the method of Smith *et al.* (13) using bovine serum albumin as a standard. On the day of assay the frozen suspensions were thawed at room temperature and treated with 0.08% Triton X-100 at an approximate protein concentration of 0.32 mg/ml by gentle stirring at 2°C for 10 min (17). Treatment was terminated by centrifugation at 50,000*g* for 20 min, and the resulting pellets were suspended in the same volume of buffer followed by one additional centrifugation-resuspension cycle.

Aliquots (0.3-0.4 mg protein) of Triton-treated membranes were incubated in triplicate at 30°C with 25 nM ^{3}H-MK-801 (17.1 Ci/mmol) and 8 concentrations of unlabeled MK-801 in the range of 1-1000 nM. The incubation occurred in 0.5 ml of 50 mM Tris acetate buffer containing glutamate and glycine at final concentrations of 20 μM and leupeptin at 87.5 μM, and was continued for 90 min to achieve binding equilibrium in the presence of amino acids. Incubation was terminated by the addition of 3 ml ice-cold buffer and subsequent filtration through Whatman GF/B glass fiber filters under a constant vacuum with a cell harvester. Filters were rapidly rinsed 4 additional times with 3 ml cold buffer, and radioactivity retained on the filters quantified by liquid scintillation spectrometry. Nonspecific binding was defined by 100 μM unlabeled MK-801 with specific binding accounting for ~90% of that found in the absence of the unlabeled ligand. Receptor binding constants were estimated from measures of specific binding at each unlabeled MK-801 concentration using LIGAND software.

The cumulative displacement curve for control membranes was fitted by nonlinear regression after normalization for maximal MK-801 binding and is shown in Figure 16. The resulting IC_{50} value is in agreement with previously reported values for this receptor and radioligand (e.g., 17-18). The LIGAND sums of squares for one- and two-site receptor regression fits clearly indicated that the binding data were best fit by a two-site model, but the nature of the low affinity site is unknown.

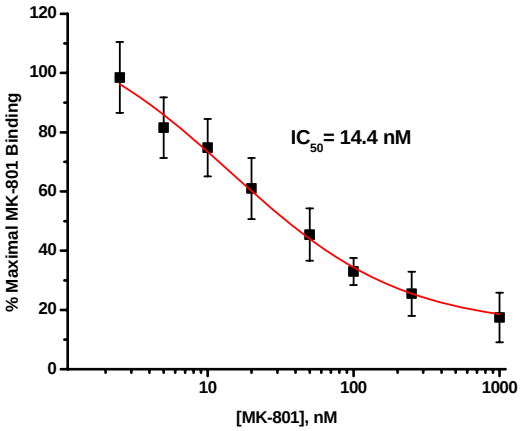

Figure 16. Percentage inhibition of specific ^{3}H-MK-801 binding in hippocampal membranes from control animals as a function of unlabeled MK-801 concentration. Cumulative data is displayed from 7 experiments conducted in triplicate. Values shown are mean ± SEM.

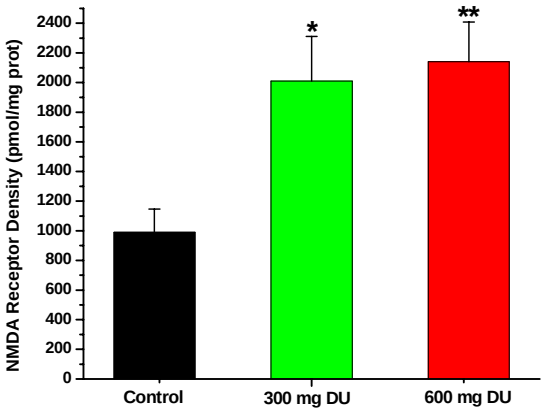

Figure 17. Density (B_{max}) of high affinity hippocampal ^{3}H-MK-801 binding sites as a function of the amount of implanted DU over 16-17 months. Mean ± SEM receptor densities are expressed in picomoles per milligram protein (pmol/mg prot)., and derived from 6-7 Scatchard analyses conducted in triplicate with each analysis based on tissue pooled from two animals. *p < 0.05; **p < 0.01 compared to control value.

As shown in Figure 17, chronic DU exposure resulted in an up-regulation of high affinity NMDA receptors by more than doubling receptor density in both the 300 and 600 mg dose groups compared to control values. There were no statistically significant changes in binding affinity for MK-801 to the NMDA receptor channel. The up-regulation of high affinity NMDA receptor sites is in contrast to reports of decreased hippocampal neuronal excitability after long-term DU exposure (2). However, since MK-801 binds to a site within the ion channel, it is not possible to distinguish whether the enhanced receptor density is due to increased expression of receptor protein or to a DU-induced conformational change in the receptor that confers greater access to the channel site for this ligand. Further studies are required to discriminate whether this up-regulation is a direct effect on the receptor or a compensatory response to presynaptic changes. Similar binding experiments are currently in progress utilizing cortical tissue to assess the selectivity of the chronic DU effect on hippocampal NMDA receptors, but results are not available at this time.

Taken together, the findings from the acute and chronic DU exposure studies suggest the following pattern of actions. When acutely applied in superfusion media, UO_2^{+2} potently diminishes depolarization-evoked glutamate release in a manner similar to that of other divalent metal ions, suggesting inhibition of Ca^{+2} influx through voltage-sensitive channels. However, when tissue is exposed *in vivo* over an extended period of time more complex mechanisms of action evolve. It is plausible to suggest that DU is sequestered into glial cells and subsequently inhibits glutamate/GABA reuptake, possibly through disruption of mitochondrial energy production. Glutamate and GABA are preferentially affected because termination of their action is dependent on transport into astrocytes moreso than for other neurotransmitters. The inhibitory actions of UO_2^{+2} on glutamate release are either no longer present or are obscured by the enhanced extracellular levels of the transmitter. Thus, the increased responsiveness to K^+ stimulation may not necessarily represent a larger magnitude of exocytosis, but may nonetheless elevate the synaptic noise level so that neurotransmission is less efficient. The NMDA receptor up-regulation may be a compensatory response to the voltage-sensitive channel blocking properties of UO_2^{+2} or the diminished signal/noise ratio, or could be a reflection of a conformational change in the receptor channel induced by chronic DU exposure. Alternatively, this latter phenomenon could be the basis of the low affinity MK-801 binding site.

A chronic neurotoxicant exposure that results in elevated CNS extracellular glutamate concentrations and up-regulated NMDA receptors by whatever mechanism may predispose the individual to excitotoxicity and increased formation of reactive oxygen species (ROS), and these mechanisms have been cited as components of the pathogenesis of neurological diseases such as amyotrophic lateral sclerosis (ALS) (19). Moreover, an increased incidence of ALS mortality has been reported in men serving in the military in previous conflicts (20). Thus, the mechanisms underlying chronic DU neurotoxicity may unexpectedly be of general importance for military health.

The findings from the acute exposure studies and the exposure protocol characterization work are reported in two manuscripts currently under review. The experiments utilizing chronic exposure have only recently reached completion, and the results were reported for the first time at the Military Health Research Forum San Juan in May, 2006. Manuscripts are currently in preparation for these latter studies. The results presented in this report clearly demonstrate a

good level of productivity, and at least 4-5 papers are resulting from the project and will make a significant contribution to the DU literature.

Problems Encountered and Addressed

The original experimental design for the project stipulated the use of intracerebral microdialysis to determine the effects of chronic DU exposure on stimulated hippocampal glutamate and GABA release. The appeal of this procedure was its ability to assess the effects of chronic treatments on CNS extracellular fluid neurotransmitter concentrations in awake animals, but significant problems were encountered in implementing these studies. Two small cohorts totaling 25-30 implanted animals were expended in initiating these studies before it became unequivocally apparent that an alternate approach was necessary. Surprisingly, the key factors were strain and age of the test animals at completion of the DU exposure period. An animal implanted at 2-4 months of age and exposed for a year was 14-16 months old at the time of stereotaxic surgery. This was not anticipated to be a problem based on previous successful utilization of the methodology in Long-Evans rats as old as 20-21 months of age (14), and the absence of problems in 3-4 month old Sprague-Dawley pilot animals. However, older Sprague-Dawley rats exhibited a low level of persistent intracranial bleeding after cannula implantation. This bleeding was not hemorrhagic nor of sufficient magnitude to produce stroke-like conditions, but frequently flowed into the cannulae and dried, preventing proper insertion of the microdialysis probe on test day. Alternatively, blood-borne glutamate was sufficient to contaminate extracellular fluid determinations at the tissue implant site. In response to these problems cannula implant sites were moved laterally away from the midline venous sinus, inserted at angles to the surface of the skull, and directed to the original tissue sites, but the problem was not substantially diminished.

A scientifically valid and acceptable alternative was to prepare transverse dorsal hippocampal slices for stimulation in superfusion chambers as an *ex vivo* model of the cellular milieu in the intact animal. While the experimental hypothesis was successfully tested in this manner (see Figures 12-15), the cost of replacing these animals and DU pellets prevented conduct of the glutamatergic AMPA receptor binding studies. It was considered of greater importance to obtain measures of stimulated release from chronically exposed animals than to determine alterations in binding to this receptor in these rats.

Also, experiments utilizing acute exposure *in vitro* based on free UO_2^{+2} ion concentrations had been proposed to permit a more systematic and reliable definition of the actions of this ion on synaptosomal glutamate/GABA release and NMDA receptor binding. However, a chelating agent that produced consistent results under these experimental conditions was not identified. Both citrate and *trans*-1,2-diaminocyclohexane-*N*,*N*,*N'*,*N'*-tetraacetate (CDTA) chelating agents and corresponding stability constants for UO_2^{+2} were found in the literature (21), but consistent and reliable results in preliminary studies were not obtained. This could have been due to the limited correspondence between the physiological buffers used in this project and the solution parameters in which the stability constants were measured. Alternatively, the complex equilibria of uranium in solution may have invalidated the assumptions underlying the metal-chelating agent model system. Additional investigations are necessary to resolve these issues.

KEY RESEARCH ACCOMPLISHMENTS

The key research accomplishments on this project were:

- Standardization of DU/tantalum pellet preparation and implantation procedures
- Establishment of chronic DU exposure protocol in adult rats with blood and brain uranium concentrations
- Determination of a differential potency for acute uranium exposure *in vitro* to diminish stimulated glutamate and GABA release, and identification of the potency of nominal uranium concentrations as similar to that of other multivalent metal ions
- Demonstration of increased glutamate and GABA release in response to depolarization in hippocampal slices from chronically exposed animals; the effect is observable in the presence or absence of Ca^{+2}
- Determination of a two-fold up-regulation of high affinity NMDA receptor density in hippocampus of both groups of chronically DU exposed animals

The latter three accomplishments represent novel observations of the components of DU neurotoxicity, and create a context for evaluating other neurochemical and neurophysiological findings.

REPORTABLE OUTCOMES

Abstracts and Presentations

Lasley, S.M. and Vietti, K.R. Acute exposure to uranium decreases potassium-stimulated hippocampal glutamate release. The Toxicologist 78, 1140, 2004.

Lasley, S.M. and Vietti, K.R.N. Acute exposure to uranium *in vitro* decreases potassium-stimulated hippocampal glutamate release. Military Health Research Forum, San Juan, Puerto Rico, April 25-28, 2004.

Lasley, S.M. and Vietti, K.R.N. Acute exposure to uranyl ion (UO_2^{+2}) *in vitro* diminishes K^+-stimulated glutamate/GABA release independent of extracellular calcium. The Toxicologist 90, 628, 2005.

Lasley, S.M., Wang, L.C. and Vietti, K.R.N. Chronic exposure to depleted uranium enhances K^+-stimulated hippocampal glutamate release in rats. Military Health Research Forum, San Juan, Puerto Rico, May 1-4, 2006.

Manuscripts Submitted for Publication

Vietti, K.R.N. and **Lasley, S.M.** Long-term exposure to depleted uranium via intramuscular implants in rats: Accumulation in blood and brain tissue. Submitted.

Lasley, S.M. and Vietti, K.R.N. Acute exposure to uranium *in vitro* differentially diminishes K^+-stimulated hippocampal glutamate and GABA release. Submitted.

Two other manuscripts are currently in preparation.

Additional Grant Applications Resulting from This Work
PR064534 – "Biochemical and Neurophysiological Bases of Developmental Neurotoxicity from Chronic Depleted Uranium Exposure", S. M. Lasley, Ph.D., PI; Peer-Reviewed Medical Research Program, USAMRMC, $968,750 direct costs for entire project, 12/06 – 11/10

CONCLUSIONS

Blood and hippocampal DU concentrations in chronically exposed animals increase monotonically up to 12 months exposure duration at the exposure levels utilized in this work. In addition, a small but significant impairment in the rate of somatic growth is present in the exposed groups compared to controls. Notably, the hippocampal uranium levels measured by ICP-MS are devoid of the apparent sample contamination found in other reports, as indicated by the consistently low DU values observed in animals implanted with tantalum pellets.

Acute uranium exposure *in vitro* clearly produces a differential inhibitory effect on potassium-stimulated Ca^{+2}-dependent glutamate and GABA release in hippocampal synaptosomes, exhibiting a substantially more potent effect on the glutamatergic process. The IC_{50} (1.92 μM) for nominal uranium concentrations on glutamate release is remarkably similar to values determined for other multivalent metal ions on transmitter release, suggesting that uranium also possesses developmental neurotoxicant properties. On the other hand, acute exposure to uranium *in vitro* does not appear to result in Ca^{+2}-mimetic actions on K^+-stimulated glutamate or GABA release.

Chronic DU exposure produces an enhancement of stimulated glutamate and GABA release compared to control values. This enhancement is greater in the presence of calcium in the superfusate, but present also in its absence. These increases are primarily observable in the 600 mg DU dose group, but also can be seen in the 300 mg dose group with respect to glutamate release. Chronic exposure also results in an up-regulation of high affinity NMDA receptors by more than doubling receptor density in both the 300 and 600 mg dose groups compared to control values. The MK-801 displacement curves are best fit by a two-receptor site model, but the nature of the low affinity site is not known.

The findings from the acute and chronic DU exposure studies suggest the following pattern of actions. When acutely applied in superfusion media, UO_2^{+2} potently diminishes depolarization-evoked glutamate release in a manner similar to that of other divalent metal ions, suggesting inhibition of Ca^{+2} influx through voltage-sensitive channels. However, when tissue is exposed *in vivo* over an extended period of time more complex mechanisms of action evolve. It is plausible to suggest that DU is sequestered into glial cells and subsequently inhibits glutamate/GABA reuptake, possibly through disruption of mitochondrial energy production. Glutamate and GABA are preferentially affected because termination of their action is dependent on transport into astrocytes moreso than for other neurotransmitters. The inhibitory actions of UO_2^{+2} on

glutamate release are either no longer present or are obscured by the enhanced extracellular levels of the transmitter. Thus, the increased responsiveness to K^+ stimulation may not necessarily represent a larger magnitude of exocytosis, but may nonetheless elevate the synaptic noise level so that neurotransmission is less efficient. The NMDA receptor up-regulation may be a compensatory response to the voltage-sensitive channel blocking properties of UO_2^{+2} or the diminished signal/noise ratio, or could be a reflection of a conformational change in the receptor channel induced by chronic DU exposure.

The value of this knowledge as a scientific product resides in the elucidation of the altered synaptic processes underlying neurotoxicity from chronic DU exposure. Evidence has been uncovered that suggests a direct effect of uranium to diminish stimulated hippocampal glutamate release, which may account for the reported decrease in neuronal excitability (3). However, other findings indicate that long-term DU exposure may also inhibit glial glutamate and GABA uptake and up-regulate NMDA receptors, possibly predisposing the individual to excitotoxicity and neurodegenerative disease. The establishment of the DU chronic exposure protocol as a shrapnel wound model based on blood and brain levels of the metal and altered rates of growth is also notable. These measures provide benchmark values for future studies and for correlation of results from this project to those obtained in other laboratories. Given the similarity of the effects of uranium on transmitter release to those of other multivalent metals (e.g., methylmercury, lead) and the fact that exposure in military scenarios is continuing, it is clear that additional studies are warranted on uranium's actions, particularly those related to developmental neurotoxicity.

REFERENCES

1. McDiarmid, M.A., Keogh, J.P., Hooper, F.J., McPhaul, K., Squibb, K., Kane, R., DiPino, R., Kabat, M., Kaup, B., Anderson, L., Hoover, D., Brown, L., Hamilton, M., Jacobson-Kram, D., Burrows, B. and Walsh, M. Health effects of depleted uranium on exposed Gulf War veterans. *Environ. Res.* 82, 168-180 (2000).
2. Pellmar, T.C., Fuciarelli, A.F., Ejnik, J.W., Hamilton, M., Hogan, J., Strocko, S., Emond, C., Mottaz, H.M. and Landauer, M.R. Distribution of uranium in rats implanted with depleted uranium pellets. *Toxicol. Sci.* 49, 29-39 (1999).
3. Pellmar, T.C., Keyser, D.O., Emery, C. and Hogan, J.B. Electrophysiological changes in hippocampal slices isolated from rats embedded with depleted uranium fragments. *NeuroToxicology* 20, 785-792 (1999).
4. Briner, W. and Murray, J. Effects of short-term and long-term depleted uranium exposure on open-field behavior and brain lipid oxidation in rats. *Neurotoxicol. Teratol.* 27, 135-144 (2005).
5. Bussy, C., Lestaevel, P., Dhieux, B., Amourette, C., Paquet, F., Gourmelon, P. and Houpert, P. Chronic ingestion of uranyl nitrate perturbs acetylcholinesterase activity and monoamine metabolism in male rat brain. *NeuroToxicology* 27, 245-252 (2006).
6. Fitzmaurice, G.M., Laird, N.M. and Ware, J.H. *Applied Longitudinal Analysis.* Wiley & Sons, Hoboken, New Jersey (2004).
7. Pellmar, T.C., Hogan, J.B., Benson, K.A. and Landauer, M.R. Toxicological evaluation of depleted uranium in rats: Six-month evaluation point. *AFRRI Special Pub. 98-1*, vol. 14 (1998).

8. Tallarida, R.J. and Murray, R.B. *Manual of Pharmacologic Calculations with Computer Programs.* Springer-Verlag, New York (1987).
9. Say, R., Ersoz, A. and Denizli, A. Selective separation of uranium containing glutamic acid molecular-imprinted polymeric microbeads. *Separation Sci. Tech.* 38, 3431-3447 (2003).
10. Tomsig, J.L. and Suszkiw, J.B. Metal selectivity of exocytosis in α-toxin-permeabilized bovine chromaffin cells. *J. Neurochem.* 66, 644-650 (1996).
11. Svehla, G. In: *Vogel's Qualitative Inorganic Analysis*, 7th ed., pp. 303-310. Singapore, Longman (1996).
12. Sutton, M. and Burastero, S.R. Uranium(VI) solubility and speciation in simulated elemental human biological fluids. *Chem. Res. Toxicol.* 17, 1468-1480 (2004).
13. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K.,Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76-85, 1985.
14. Lasley, S.M. and Gilbert, M.E. Rat hippocampal glutamate and GABA release exhibit biphasic effects as a function of chronic lead exposure level. *Toxicol. Sci.* 66, 139-147 (2002).
15. Aschner, M., Yao, C.P., Allen, J.W. and Tan, K.H. Methylmercury alters glutamate transport in astrocytes. *Neurochem. Intl.* 37, 199-206 (2000).
16. Allen, J.W., Mutkus, L.A. and Aschner, M. Methylmercury has a selective effect on mitochondria in cultured astrocytes in the presence of [U-^{13}C]glutamate. *Brain Res.* 902, 92-100 (2001).
17. Yoneda, Y., Ogita, K. Labeling of NMDA receptor channels by [^{3}H]MK-801 in brain synaptic membranes treated with Triton X-100. *Brain Res.* 499:305-314, 1989.
18. Lasley, S.M., Green, M.C. and Gilbert, M.E. Rat hippocampal NMDA receptor binding as a function of chronic lead exposure level. *Neurotoxicol. Teratol.* 23, 185-189 (2001).
19. Carri, M.T., Grignaschi, G. and Bendotti, C. Targets in ALS: Designing multidrug therapies. *Trends Pharmacol. Sci.* 27, 267-273 (2006).
20. Ascherio, A. Military service and ALS mortality in the Cancer Prevention Study-II. *Military Health Research Forum*, San Juan, Puerto Rico (2006).
21. Smith, R.M. and Martell, A.E. *Critical Stability Constants*. Plenum Press, New York (1976).